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REVIEW

Olfactory Processing Pathways of the Insect Brain

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INTRODUCTION

In insects, olfaction plays a major role in such behaviors as mating, feeding, and oviposition, which are all essential for maintaining the species. For many insects, including moths, conspecific sex pheromones are important stimuli for reproductive behaviors. Like males of many other species of moths, the male Sphinx moth *Manduca sexta* responds to the sex-pheromone blend released by conspecific females with a characteristic sequence of behaviors, including upwind flying with a zig-zag pattern [1, 2]. These pheromones stimulate highly sensitive and selective receptor cells associated with particular classes of male specific sensilla on the antennal flagellum [3–8].

These receptor cells [6], in the form of patterned firing of impulses, transmit information about pheromones along the axons of the receptor cells to the primary order olfactory center, the antennal lobes (ALs) of the brain [9–17]. In the central nervous system (CNS), this olfactory information passes through several stages of synaptic processing in the ALs and higher centers in the protocerebrum (PC), where it is integrated with sensory information of other modalities; e.g., visual and mechanosensory [18]. Then, via "descending" premotor neurons, the integrated multimodal output of brain circuits may act on thoracic motor circuitry to affect behavior [19].

This review focuses on some of our findings and describes the functional organization of olfactory pathways throughout the brain, especially in the higher olfactory center in the protocerebrum, of *M. sexta* males.

OLFACTORY RECEPTORS ON THE ANTENNA

Sex-attractant pheromones stimulate receptor cells in a class of male-specific sensilla, the *sensilla trichodea,* on the antennal flagellum [3–6]. The male flagellum has about 2.6×10^5 sensory neurons associated with about 10^5 recognized sensilla. About 40% of these are male-specific and pheromone-sensitive *sensilla trichodea* [3–6]. Each pheromone-sensitive sensillum comprises two olfactory receptor cells in *M. sexta* [3–6]: One is sensitive to (*E,Z*)-10,12-hexadecadienal (E10, Z12-16 : AL or bombykal), the major component of the pheromones, and the other is insensitive to physiological concentrations of bombykal, but is stimulated by one of two other components of the pheromones, either (*E,E,Z*)-10,12,14-hexadecatrienal (E10,E12,Z14-16 : AL) or (*E,E,E*)-10,12,14-hexadecatrienal (E10,E12,E14-16 : AL) [6, 7]. (*E,Z*)-11,13-pentadecadienal (E11,Z13-15 : AL), which is not present in the natural pheromone blend, is a relatively stable and specific mimic of E10,E12,Z14-16 : AL for stimulation of the "EEZ" subpopulation of these latter cells [6, 7].

Numerous other olfactory receptor cells populate the antennal flagella, including receptors sensitive to plant volatiles such as the leaf aldehyde (*E*)-2-hexenal (E2-6 : AL) [8–10], as well as receptors of other modalities, including hygroreceptors, mechanoreceptors, and thermoreceptors [11].

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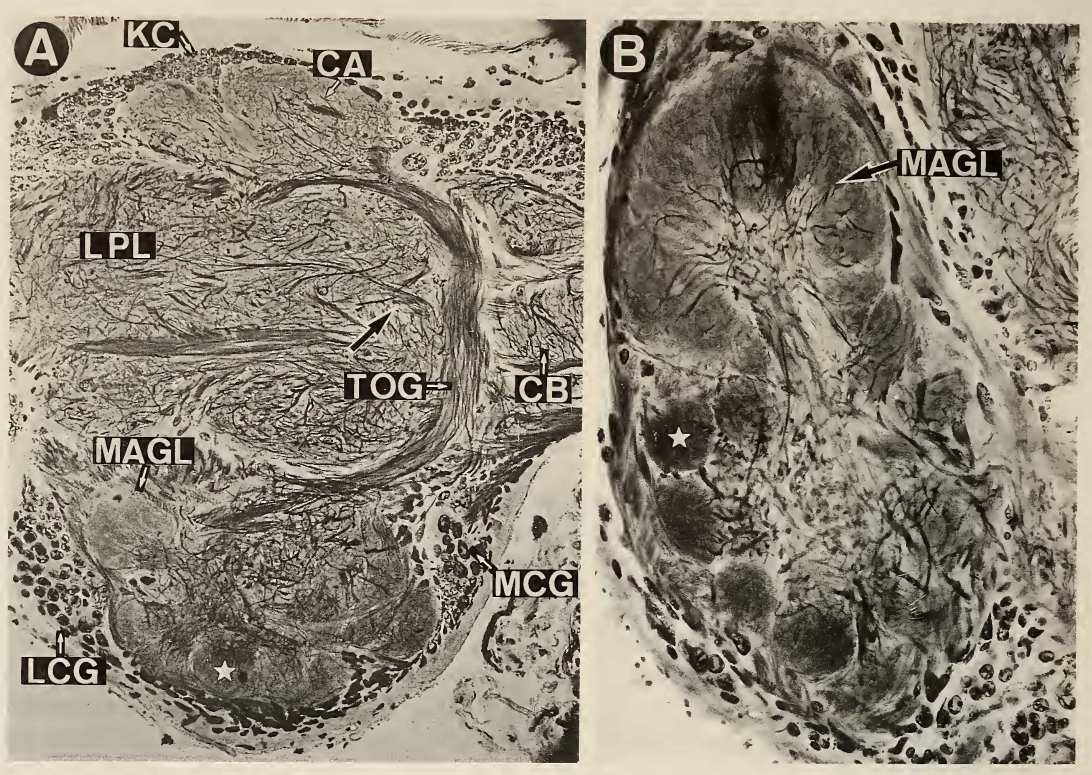


FIG. 1. The brain of the male *Bombyx mori* stained by Bodian's methods. (A) A fronto-horizontal section of the right brain. *CA* calyces of the mushroom body; *CB* central body; *KC* cell bodies of Kenyon cells; *LCG* lateral cell group; *MAGL* (=MGC) macroglomerular complex; *TOG* (=inner antenno cerebral tract; IACT) tractus olfactorio-globularis. Thick arrow: a small bundle separated from the TOG in the lateral part of the central body. ★: microglomerulus (=ordinary glomerulus) Scale bar=100 μm (B) Transversal section of the right deutocerebrum. Each hemisphere of the deutocerebrum contains one MGC in the dorsal and about 60 ordinary glomeruli in the ventral region. Females lack the MGC. Scale bar=100 μm. (Kanzaki and Shibuya [27]).

DEUTOCEREBRAL NEURONS

The deutocerebrum consists of two distinct neuropils: the antennal lobe (AL) and the antennal mechanosensory and motor center (AMMC) (Fig. 2A). Antennal olfactory receptor cells project axons into the brain via the antennal nerve, and these fibers terminate in the ipsilateral AL, the primary olfactory center in the brain [9]. Each AL of the male contains a prominent, sexually dimorphic macroglomerular complex (MGC, 300–400 μm in diameter) innervated by axons of pheromone-specific receptors (Figs. 1–3). ALs of both male and female moths also contain 64 ± 1 spheroidal 'ordinary' glomeruli [12] (50–100 μm in diameter) which receive innervation from the axons of receptors sensitive to other odors (Figs. 1, 2, 5, 6) [9, 12, 13]. On the other hand, the AMMC receives axons from mechanosensory neurons in the two basal antennal segments, the scape and pedicel; i.e., bristles; Böhm's organ [20], chordotonal organs; Johnston's organ [21] and Janet's organ [22]. The AMMC is furthermore innervated by dendrites of antennal-muscle motor neurons. Bordering the AL are three groups of AL neuronal somata (local, medial, and anterior) totalling 1200 cells [9, 23, 24].

The AL has been studied in certain Lepidoptera, including *M. sexta, Bombyx mori* (Fig. 1) [25–29] and *Antheraea polyphemus* [30], in several species of cockroaches, most prominently *Periplaneta americana* [31–34], and in honey bees [35–37], ants [38, 39] and flies [40–44].

In *M. sexta,* olfactory information is processed

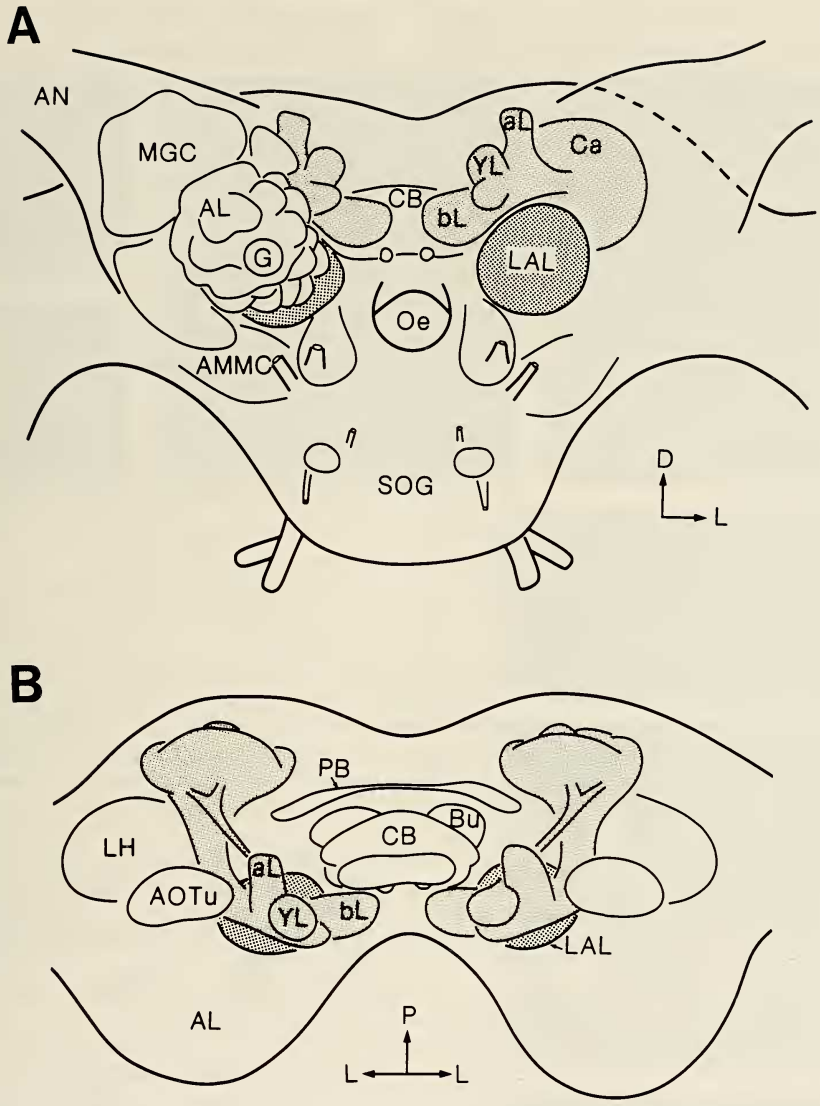


FIG. 2. Diagrams of neuropils in the mid-brain of *Manduca sexta*. (A) Dorsal view, (B) Frontal view. *aL* alpha-lobe; *AL* antennal lobe; *AMMC* antennal mechanosensory and motor center; *AN* antennal nerve; *AOTu* anterior optic tubercle; *Bu* buttress; *bL* beta-lobe; *Ca* calyces of the mushroom body; *D* dorsal; *L* lateral; *LAL* lateral accessory lobe; *Oe* oesophagus; *P* posterior; *PB* protocerebral bridge; *SOG* suboesophageal ganglion; *YL* Y-lobe.

in the AL by two morphologically distinct classes of neurons: those confined to the AL (local interneurons; LNs) and those with axons projecting from the AL to other regions of the brain (projection or output neurons; PNs) [9, 10, 23, 24, 45–47].

Several types of AL projection neurons (PNs) have been identified in *M. sexta,* distinguished by uni- (Figs. 3, 5) or multi- glomerular (Fig. 6) arborizations within the AL and axons projecting through one of five different tracts (inner, middle, outer, dorsal, and dorso-medial antennocerebral tracts) to the protocerebrum (Figs. 3, 5, 6) [10, 23, 24, 46, 47].

The AL PNs fall into two categories according to

their responses to olfactory stimuli: (1) neurons excited or inhibited by pheromones and (2) those showing no preference for pheromonal stimuli. In general, morphological differences between these two groups reinforce the physiological categorization. PNs of the first class have major dendritic arborization in the MGC (MGC PNs) and none in the ordinary glomeruli (Fig. 3) [10, 45, 48]. PNs of the second class have major arborizations in ordinary glomeruli (Gs) but not in the MGC (G PNs) (Fig. 5) [10]. Similar functional organization in AL has been demonstrated in *Bombyx* [25–29], *Antheraea* [30] and *Periplaneta* [31–34].

(1) *Macroglomerular complex projection neurons (MGC PNs)*

Pheromones delivered to the antenna elicit excitatory or inhibitory responses in ipsilateral MGC PNs (Fig. 4) [10, 45, 48]. Some MGC PNs of *M. sexta* respond to antennal stimulation with E2-6:AL. In most cases, the response elicited is an inhibition of ongoing background activity (Fig. 4) [10]. Certain central olfactory neurons in *P. americana* have similar response characteristics; that is, they are excited by a major pheromone (Periplanone B), but inhibited by plant odors [34].

Odor quality appears to be represented in the sign of the responses in some MGC PNs; i.e., excitation for E10,Z12-16:AL or female extract and inhibition for plant odor (E2-6:AL) (Fig. 4). Recordings during delivery of different concentrations of odorants to the antenna suggest that changes in the impulse frequency and the latency of the response reflect concentration changes (Fig. 4) [10, 45]. Similar properties appear to encode odorant concentration in other insect olfactory systems tested [27, 30, 34].

In *P. americana,* MGC PNs that are excited by

FIG. 3. Reconstruction of a MGC projection neuron of *M. sexta.* (A-G) Photomicrographs of 25-μm frontal sections exhibiting Lucifer-Yellow stained neurites of the injected neuron. Reconstructions were made from color slides of such sections. (A, B) Processes in the inferior lateral protocerebrum (*ILPR*). (C, D) Branches in the principal calyces of the mushroom body (*Ca*). Scale bar in (D) (for A-G)=100 μm. (E) Neurite projecting in the inner. antenno-cerebral tract (*IACT*). (F, G) Branches innervating putative partitions within the macroglomerular complex (*MGC*). (H) Exploded frontal view of the injected neuron reconstructed from serial sections including those shown in (A-G). *Dashed lines* link processes that were separated to reveal the structures of branches that overlapped *in situ. Arrowheads* mark 5 distinct 'partitions' of the MGC arborization, possibly corresponding to functionally significant partitions of the MGC itself. Scale bar=100 μm. (I) Inset shows a diagrammatic horizontal view of the ipsilateral hemisphere and projection areas occupied by the injected neuron. Other abbreviations: *AL* antennal lobe; *G* ordinary glomerulus; *cb* neuronal cell body; *P* posterior; *L* lateral. (Kanzaki *et al.* [10].

FIG. 4. Intracellular recordings from an MGC projection neuron of *M. sexta.* (A) Responses to olfactory stimulation of the ipsilateral antenna. (a, b) Female extract excited the cell as did E10,Z12-16:AL. (c, d) E2-6:AL or iso-5:OAc inhibited background firing. Breakes in the records in (c) and (d) each represent 1 sec during which no action potentials were generated. No responses were observed to olfactory stimulation of the contralateral antenna. Scales=1 sec, 20 mV. (B) Impulse-frequency histograms (number of impulses per 0.25 sec 'bin') of this neuron's responses to stimulation with different concentrations of odorants. Asterisks (*) mark responses shown in (A). (C) Dose-response relationships of 5 pheromone-sensitive neurons with antennal stimulation by E10,Z12-16:AL. Two of these neurons, marked with asterisks (*), were not characterized morphologically; the others had arborizations in the MGC. Dashed line indicates background activity. (Kanzaki *et al.* [10]).

FIG. 5. Reconstruction of an ordinary uniglomerular projection neuron of *M. sexta.* This neuron was inhibited by stimulation of the ipsilateral antenna with E2-6:AL or iso-5:OAc. (A-H) Photomicrographs of 25 μm fronto-horizontal sections exhibiting Lucifer Yellow-stained neurites of the injected neuron. (A) Branches in the LH. Scale bar for (A-E)=100 μm. (B-D) Innervation of the principal calyces of the ipsilateral mushroom body. (E) Neurite projecting in the IACT. (F-H) Major dendritic arborization occupying a single ordinary glomerulus. Fine collaterals were observed that may have extended to neighboring ordinary glomeruli (shown at arrowhead in (I)). Scale bar in (F) for (F-H)=100 μm. (I) Drawing of the injected neuron reconstructed from serial sections (in fronto-horizontal view) including those shown in (A-H). The neurite entering the ordinary glomerulus is drawn at an optical plane showing that the thick neurite invaded the glomerulus and branched into a few stout processes that gave rise to many fine, tufted branches. The same projection is drawn at a more superficial optical plane in the inset. Scale bar=100 μm. (J) Schematic horizontal view of the mid-brain showing projection areas occupied by this nouron. (Kanzaki *et al.* [10]).

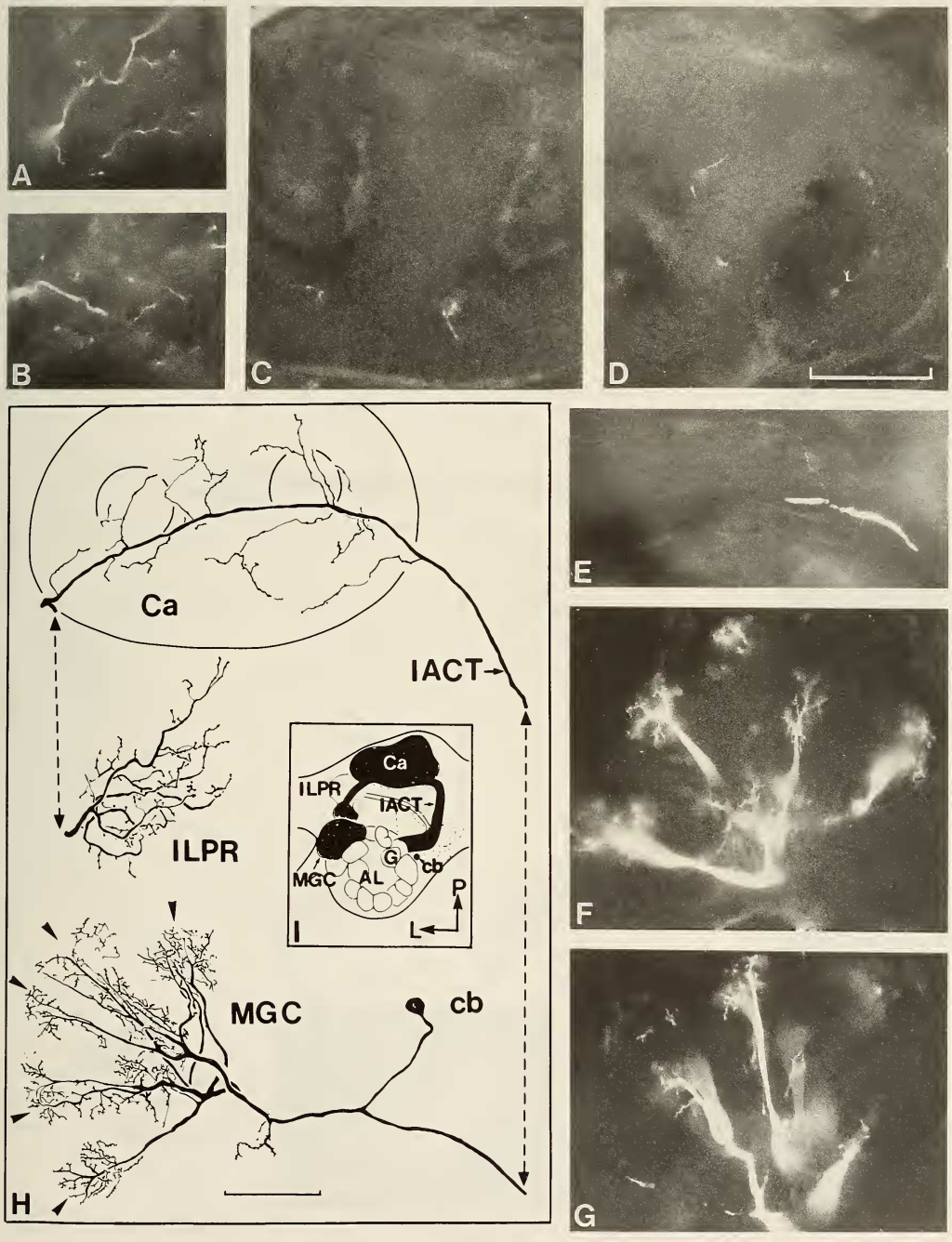
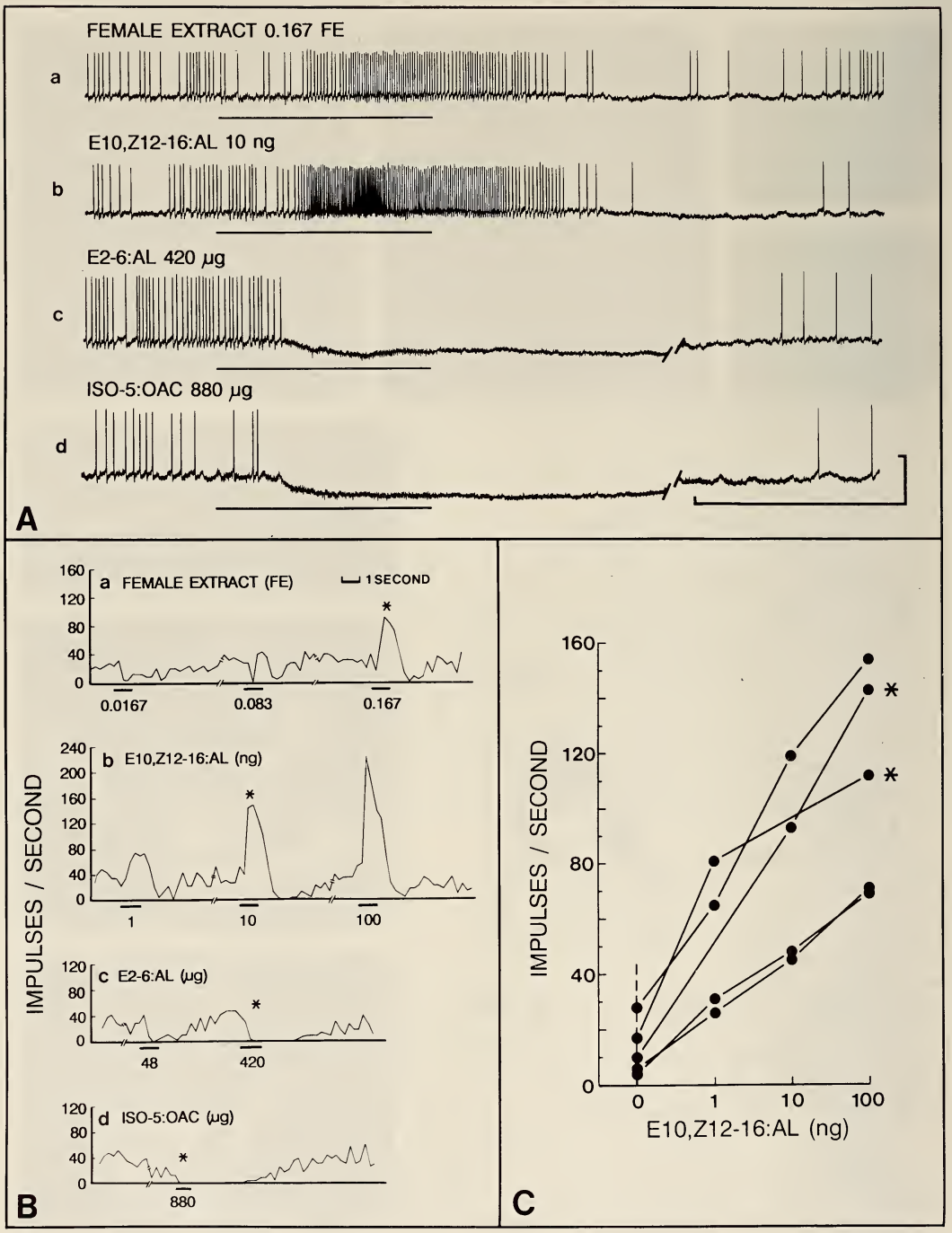


FIG. 3.


FEMALE EXTRACT 0.167 FE
a
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c
ISO-5:OAC 880 μg
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A
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0.0167
0.083
0.167
b E10,Z12-16:AL (ng)
1
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100
c E2-6:AL (μg)
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FIG. 4.

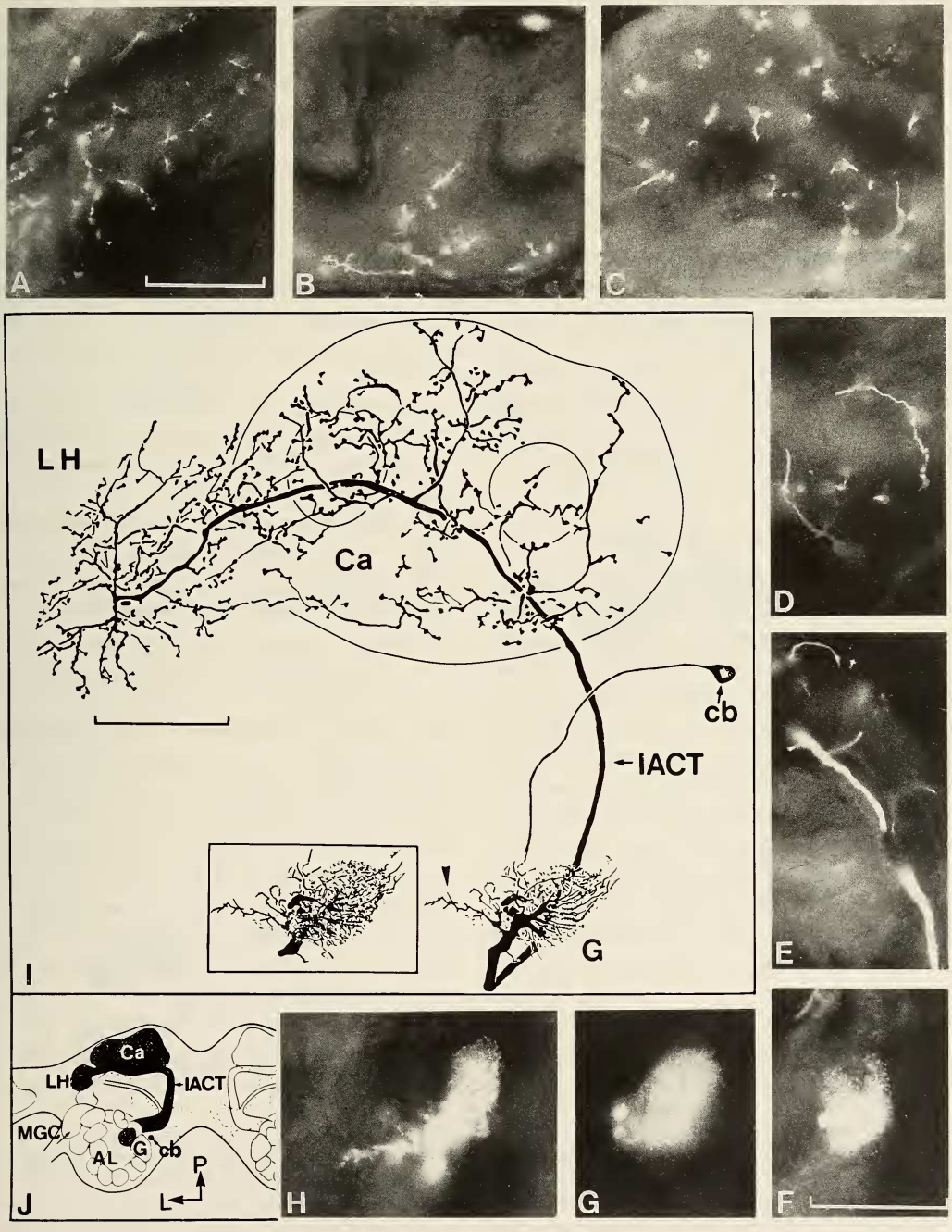


FIG. 5.

pheromones as well as plant odors have been found [34]. One example of this type of neuron has been demonstrated in *M. sexta*: a cell excited both by female extract and by E2-6 : AL [10].

(2) *Ordinary glomerular projection neurons* (*G PNs*)

Uniglomerular PNs innervating ordinary glomeruli are either excited or inhibited when E2-6 : AL or iso-amyl acetate (iso-5 : OAc) is applied to the ipsilateral antenna, but do not respond detectably to pheromones [9, 10]. Some of these uniglomerular PNs also respond to mechanosensory as well as olfactory stimuli [10]. Uniglomerular PNs showing olfactory and mechanosensory responsiveness have been demonstrated in *B. mori* (Fig. 3) [25, 27, 49], *P. americana* [31, 50], *Apis mellifera* [51], and *Achaeta domesticus* [52].

(3) *Morphology of uniglomerular PNs*

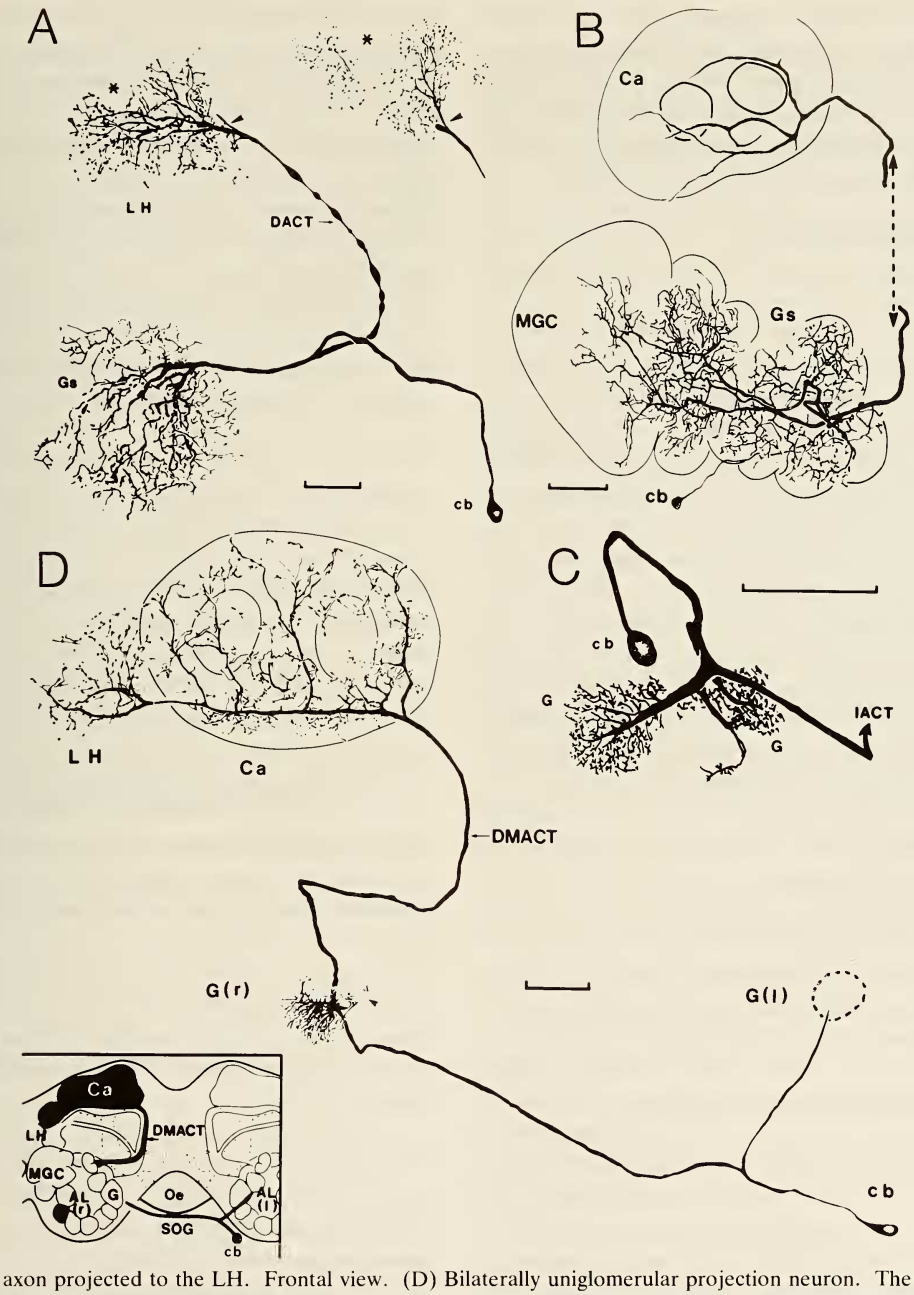
MGC PNs are so categorized because of the restriction of their dendritic arborizations to the MGC (Fig. 3). As suggested by some anatomical preparations, including Golgi- and ethyl gallate-stained sections as well as intracellularly stained cells, however, the MGC may comprise five or more distinct partitions [N. J. Strausfeld personal communication]. Some MGC projection neurons apparently have dendritic branches throughout the MGC [10, 27, 45, 48], while other MGC PNs exhibit arborizations restricted to some of the suggested divisions of the MGC (Fig. 3) [10]. Similarly partitioned MGC arborizations have been observed previously in *P. americana* [34], and *B. mori* [27].

In the first study of AL neurons of *M. sexta*, Matsumoto and Hildebrand [9] classified ordinary glomerular PNs on the basis of the appearance of their arborizations in the glomeruli. The arborization of a 'type-I output neuron' was described as largely confined to the outer part of the glomerulus and the arborization of a 'type-II output neuron', as filling its glomerulus. Such ordinary glomerular PNs are now thought to represent extremes of a variety of patterns of intraglomerular arborization exhibited by ordinary glomerular PNs [10, 23]. It was recently reported by Hansson *et al.* that MGC may be classified further into structural and functional categories based on responsiveness to pheromone components and branching in a doughnut-shaped neuropil structure, the "toroid" and a globular structure, the "cumulus" regions of the MGC [48].

Axons of both MGC and G PNs project to the lateral protocerebrum through the inner antennocerebral tract (IACT) [23], which runs under the calyces of the mushroom body (Figs. 3, 5). The calyces receive innervation from all uniglomerular MGC PNs and G PNs. Although these neurons have extensive branches within the principal calyces, the patterns of branching of G PNs are widespread in comparison with those of MGC PNs, which appear to terminate in specific zones of calycal neuropil (Figs. 3, 5) [10, 23]. In other insects, such as *A. mellifera* [37] and *Sphinx ligustri* [53], the branching of Kenyon cells within the calyces has been shown to be quite restricted. This might also be the case in *M. sexta*, a relative of *S. ligustri*, with the consequence that Kenyon cells receiving synaptic inputs from MGC PNs and G PNs may be members of two distinct populations.

Just as the branching patterns of the axon collaterals of MGC PNs and G PNs within the principal calyces differed, so also do the axon terminals of these neurons within the lateral protocerebrum. Axons of MGC PNs terminate in the inferior lateral protocerebrum (ILPR) and are spatially

Fig. 6. Reconstruction of several types of multiglomerular projection neurons of *M. sexta*. (A) Antennal lobe (*AL*) branches were evenly distributed among the ordinary glomeruli. Overlapping arborization in the lateral horn (*LH*) are drawn separately in two parts. The right drawing shows a view of the LH branches more ventral than the left drawing. The two parts overlap at the arrowheads. No branches were observed within the calyces of the mushroom body. Fronto-horizontal view. (B) Major dendritic branches occupy many or all of the ordinary glomeruli of the AL as well as branches that entered the ventral MGC. Some branches of the axon of this neuron penetrated the principal calyces of the ipsilateral mushroom body, while others projected to the superior protocerebrum (*SPC*) anterior to the calyces. Frontal view. (C) Substantial dendrites invaded at least 2 ordinary glomeruli. Branches of the axon clearly innervated the principal calyces of the ipsilateral mushroom body, and



the axon projected to the LH. Frontal view. (D) Bilaterally uniglomerular projection neuron. The cell body, located in the suboesophageal ganglion (*SOG*), sent out one branch that projected to a single ordinary glomerulus in the AL ipsilateral to the cell body and a second, long branch that extended to the AL contralateral to the cell body. A prominent dendritic arborization occupied a single ordinary glomerulus in that contralateral AL. What appeared to be the axon of this neuron was restricted this contralateral side of the brain and projected into the protocerebrum via the dorsomedial antenno-cerebral tract (*DMACT*), where the axon sent out collaterals that heavily innervated the principal calyces of the mushroom body and terminated with complex ramifications in the LH. Fronto-horizontal view. Inset: Diagrammatic horizontal view of mid-brain showing projection area of this bilaterally uniglomerular projection neuron (D). The relationship between the SOG and the brain is displaced to illustrate schematically the areas occupied. Scale bars (A-D) = 100 μm. (Modified after Kanzaki *et al.* [10]).

separated from the termination sites of G PNs axons (Fig. 3). The latter are situated more laterally and posteriorly in the lateral horn (LH) (Fig. 5). These findings suggest that the ILPR is an important site for higher-order processing of pheromonal information from the AL [10, 23].

(4) *Multiglomerular PNs*

Although uniglomerular PNs have been studied extensively in a number of insect species [9, 23, 31, 45, 48, 49, 51, 54, 55], multiglomerular PNs have been encountered less frequently [23, 56]. In *M. sexta,* some multiglomerular PNs branch sparsely throughout most of the ordinary glomeruli. Other multiglomerular PNs innervate only a few ordinary glomeruli, but branch densely within them (Fig. 6C) [10, 23]. The arborizations of the former PNs within their glomeruli resemble those of certain LNs ("type I" or L(G)) [9, 24]. Although their dendritic branching patterns within innervated glomeruli appear to be similar, the responsiveness of the LNs and PNs is quite different [9, 10]. PNs that have dendritic arborizations both in multiple Gs and the MGC, a projection pattern similar to that of certain LNs ("Type II" or L(MGC/G)) (Fig. 6B) [9, 24], are excited both by pheromones and E2-6 : AL. This pattern of responsiveness is similar to that reported for a subset of LNs with similar morphology ("Type IIa") [9]. Another type of multiglomerular PN has an axon in the dorsal antenno-cerebral tract (DACT), a tract shared by only about 45 other axons (Fig. 6A) [10, 23]. Similar to other PNs, it projects to the LH but not the calyces. This neuron also has a rather unusual feature in that its soma is located near the medial cell group of the AL contralateral to the cell's arborizations and projections. A multiglomerular neuron with a soma contralateral to its major AL branching has been recently found in *B. mori* (R. Kanzaki and T. Shibuya, unpublished observations). This neuron has no branches in the MGC but is excited by (*E,Z*)-10,12-hexadecadienol (E10,Z12-16 : OH or bombykol), the major sex pheromone of *B. mori.*

(5) *Bilaterally uniglomerular PNs*

Most central olfactory neurons characterized both morphologically and physiologically in previous studies have their major arborizations restricted to one side of the brain and show responses only to stimulation of the antenna ipsilateral to those arborizations. Nevertheless, integration of bilateral olfactory information may be important for the insect. It is thus necessary to determine at what level contralateral or bilateral information may enter the olfactory pathway in each half of the brain.

One bilaterally uniglomerular PN has been demonstrated in *M. sexta,* which is unique among AL PNs observed to date in having dense arborizations in a single ordinary glomerulus on each side of the brain (Fig. 6D). The axon of this PN is located in the dorsal medial antenno-cerebral tract (DMACT) [10, 23]. Evidence for the presence of such neurons was previously obtained in a morphological study by Kent [57]. The single neuron characterized here is inhibited by olfactory stimulation of the antenna contralateral to the soma (ipsilateral to its major arborizations) and is unaffected by olfactory stimulation ipsilateral to the soma [10]. By contrast, gentle mechanosensory stimulation of either antenna influences the firing of this neuron; contralateral stimulation is excitatory and ipsilateral stimulation, inhibitory [10]. These results indicate that convergence of modalities as well as bilateral information can enter the antennal-sensory pathway at the level of AL PNs [10].

(6) *Olfactory processing in the AL PNs*

Kanzaki *et al.* [10] proposed that several types of information may be processed by AL projection neurons in *M. sexta*:

1) Pure olfactory information. Information about pheromones appears to be selectively transmitted by MGC PNs (Figs. 3, 4) [10, 45].

Excitatory or inhibitory olfactory responses are elicited by non-pheromonal odors (e.g., E2-6 : AL or iso-5 : OAc) in ordinary glomerular projection neurons (Fig. 5). Many neurons of this type are insensitive to stimuli of other modalities (e.g., visual, mechanosensory stimulation to the antenna). Others belong to the next category.

2) Mechanosensory-modulated olfactory (or olfactory-modulated mechanosensory) information. This type of information seems, at present,

to be restricted largely to ordinary glomerular projection neurons. One neuron investigated was found to innervate multiple ordinary glomeruli (Fig. 6C) and responded to mechanosensory stimulation of the antenna but not to any odors tested. It is conceivable that this neuron transmits only pure mechanosensory information from the AL to the protocerebrum, but it seems more likely that olfactory responses would have been recorded from such a cell if other odors had been tested.

Convergence of modalities as well as bilaterally mechanosensory information can enter the antennal-sensory pathway at the level of AL PNs (Fig. 6D) [10].

3) Odor concentrations. Response latencies and impulse frequencies during excitatory responses, as well as the durations of periods of imulse suppression during inhibitory responses (the latter probably reflecting increased activation of one or more inhibitory interneurons), appear to encode quantitative information about olfactory stimuli for transmssion to the protocerebrum (Fig. 4).

PROTOCEREBRAL NEURONS

Many olfactory protocerebral (PC) neurons innervate a neuropil region lateral to the central body on each side of the PC, the lateral accessory lobe (LAL; Figs. 2, 7). Some PC neurons provide the anatomical substrate for linking the LAL to the lateral PC, a region receiving axons of antennal lobe projection neurons (e.g., Figs. 3, 5, 6). The LAL appears to be an important region of convergence of olfactory neurons from other regions of the PC [10, 18, 19].

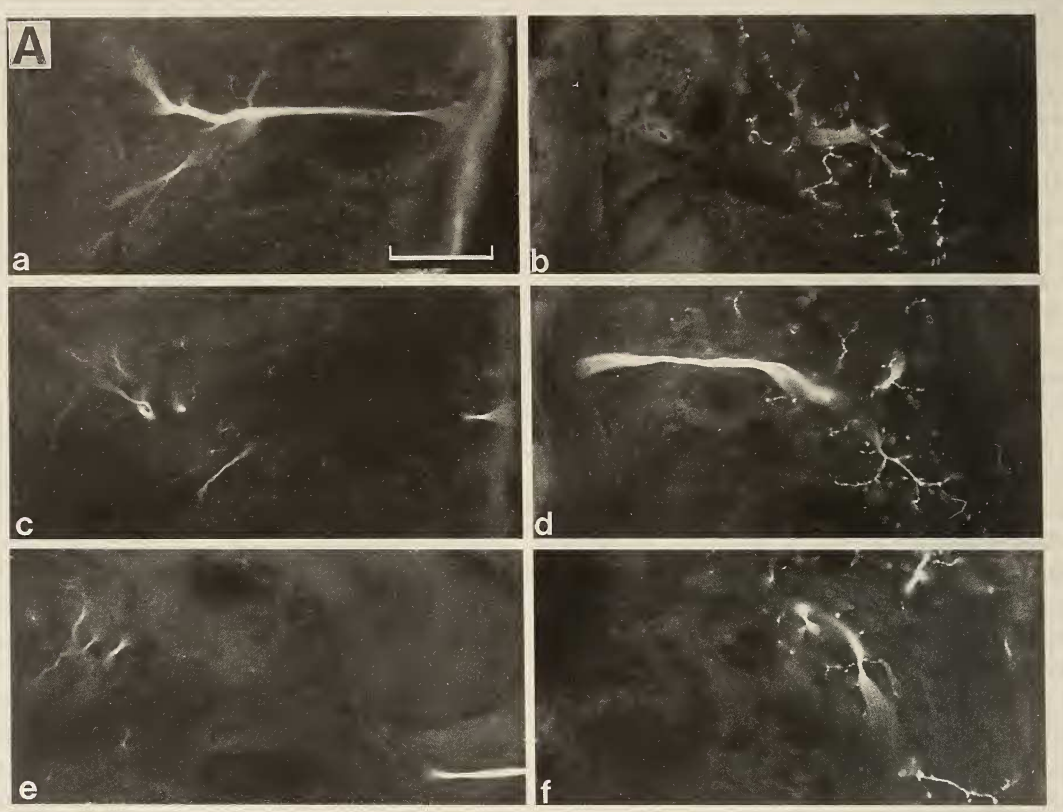
(1) *Physiology of PC neurons*

In *M. sexta,* PC neurons responding to olfactory stimulation of the antenna fell into two physiological classes: (1) those showing long-lasting excitation (LLE) (Fig. 8) and (2) those showing brief excitation (BE) in response to odor stimuli (Fig. 13). We define LLE as any odor-mediated increase in firing that persists within 30% of peak level for $\geq$1 sec beyond the period of delivery of the olfactory stimulus. BE refers to increased firing which recovers to background firing levels $<$1 sec after the stimulation. BE is similar to responses observed in antennal lobe neurons of *M. sexta* [10, 45, 48] and other species (e.g., *B. mori*-[49]), while LLE is apparently a property of some PC neurons but not antennal lobe neurons. The sex-pheromone blend induced LLE robustly in preparations in which other odors, including individual pheromone components, fail to do so (Fig. 8).

(2) *LLE responses in PC neurons*

Where does LLE arise within the olfactory pathway? LLE has not yet been encountered in intrinsic neurons or projection neurons of the antennal lobe in reported samples, exceeding 500 local neurons and 130 projection neurons studied in *M. sexta* (e.g., Fig. 4A) [9, 10, 45, 48]. Prolonged increases of firing rate have occasionally been recorded in antennal lobe neurons of other moths (*B. mori*-[27], Fig. 4; *Holiothis zea*-[58], Fig. 6), but those responses did not remain near the peak firing level for $\geq$1 sec. Of neurons in *M. sexta* that appear to receive input within the lateral PC, a prominent target area for axons of antennal lobe projection neurons [10, 23, 45], none has been shown to exhibit LLE [18]. We found LLE to be associated with neurons that innervate the LALs (Fig. 7) and MBs, and as described later, LLE in neurons appeared to be synaptically downstream from these areas (Fig. 9) [19]. Although we cannot, at present, exclude the possibility that LLE was consequence of ascending influences in our experiments, similar LLE-like responses have been recorded in preparations of *B. mori* in which the ventral nerve cord was severed ([59], Kanzaki-unpublished observations). Thus, it is likely that LLE is a response arising at the level of the MBs and/or the LALs in the protocerebrum.

LLE responses similar to those for *M. sexta* have also been observed in some MB extrinsic neurons and other PC neurons of *Apis mellifera* [51, 60], *Acheta domestica* [52], and *B. mori* [29, 49]. Previous workers have speculated that prolonged excitation might be produced by reciprocal synapses among intrinsic neurons of the MB [61] or by feedback loops from the lobes to the calyces of the MB by extrinsic MB neurons [52]. Among other mechanisms that must also be considered are: (1)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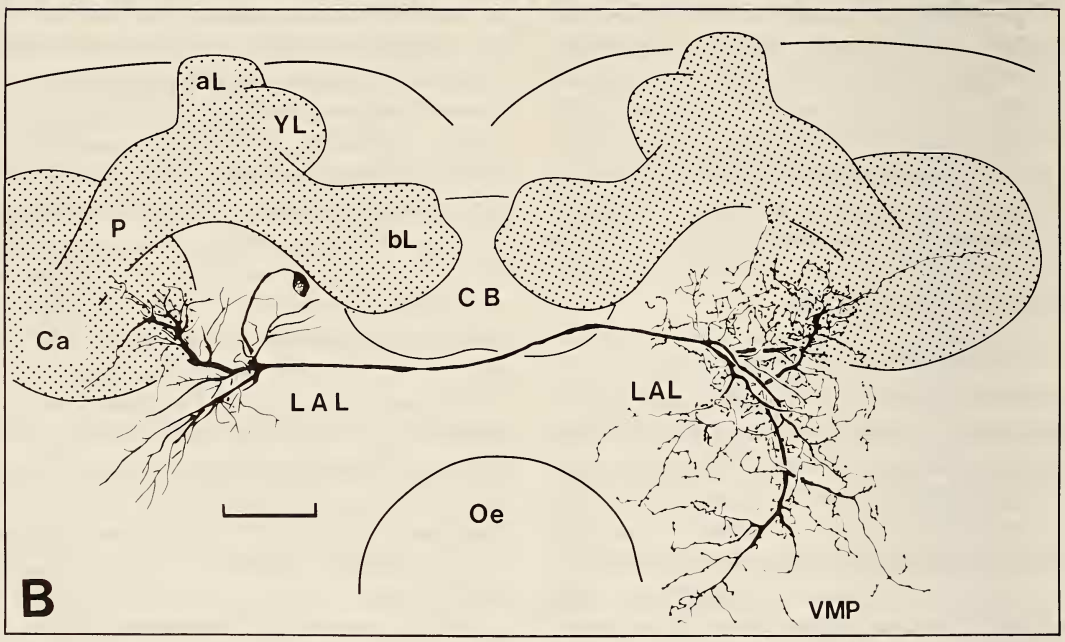
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LAL
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VMP
B

the possibility of plateau properties, similar to those demonstrated to cause prolonged firing in other types of neurons in invertebrates and vertebrates [e.g., 62–68], intrinsic to neurons at the source of LLE, and (2) other long-lasting synaptic or modulatory mechanism that increase excitability [e.g., 67, 68]. It is possible that LLE originates within the MB and is induced synaptically in subsequent neurons within the LALs or *vice versa*, or that it is independently generated at both sites. Moreover, the possibility exists that LLE is generated in some other region of the CNS and transmitted to both the MB and the LAL. Findings to date do not permit the elimination of any of these possibilities.

(3) *Integration of bilateral olfactory information at LALs in PC*

Only stimulation of the antenna ipsilateral to the soma of a bilateral LAL neuron (Fig. 7) elicits excitation [18]. This is consistent with the observation that antennal-lobe projection neurons resposive to pheromonal stimuli also respond only to ipsilateral stimulation (Fig. 4) [10, 45] and suggests that pheromonal information is integrated unilaterally within the brain and may not cross the midline before at the level of the LALs [18]. This contrasts with processing of other types of information. For example, bilateral mechanosensory information enters the pathway at the level of certain antennal-lobe projection neurons (Fig. 6D) [10]. Bilaterally uniglomerular antennal-lobe neurons (Fig. 6D) have been described which could provide the substrate for integration of bilateral information [10]. One such cell was found, however, to be sensitive to olfactory stimulation of only one antenna even though it was bilaterally mechanosensory [10].

The structure of the bilateral LAL neurons (Fig. 7) supports the idea of polarized information flow from the side ipsilateral to the soma to the contralateral side. Ipsilateral branches typically exhibited smooth profiles, while contralateral branches were distinctly different in bearing numerous varicosities (Fig. 7) [18]. Similar differences in structure have been observed in other brain neurons of *M. sexta* [46]. Although the notion requires verification with electron microscopy, it is possible that this polarity of structure is similar to that observed in crayfish neurons, where smooth profiles were shown to be exclusively post-synaptic (i.e., the input side of the neuron) and the blebby, vericose profiles presynaptic (i.e., the output side of the neuron) [69]. In further support of the idea that pheromonal information from both antennal lobes is collected at the level of the LALs is the observation that descending neurons, which have branches within the LAL and are probably downstream in the path of information flow, are sensitive to stimulation of either antenna and do not

Fig. 7. Morphology of a bilateral LAL protocerebral neuron of *M. sexta*. (A) (a-f) Photomicrographs of 25-μm frontal sections exhibiting Lucifer Yellow-stained neurites of the injected neuron. Reconstructions were made from color slides of such sections. (a, c, e) Processes ipsilateral to the cell body has smooth profiles. (b, d, f) Branches contralateral to the cell body exhibited numerous varicosities. (B) Reconstruction of the stained neuron. The bilateral arbors lay anterior to but did not penetrate the calyces (*Ca*) of the MBs (stippled). *P* peduncle; *VMP* ventro-medial protocerebrum. Scale bars = 100 μm. (Kanzaki *et al.* [18]).

Fig. 8. (A) Intracellular recordings from a bilateral LAL protocerebral neuron that responded with LLE. Clean air (a) or odorants (b-f) were delivered to the antenna as 1-sec puff stimuli, indicated by a solid line below each trace. (a) Clean air (i.e., gentle mechanical stimulation) appeared to cause a brief inhibition of firing. (b) A puff of pheromone blend (0.20 FE) elicited LLE that persisted for over 8 sec (the two traces represent a continuous record). (c, d) Individual pheromone components delivered at rather high concentrations produced much weaker responses, while tobacco-leaf odor caused brief inhibition similar to the apparent mechanosensory component revealed by blank stimulation (e). Stimulation of the antenna even with very high concentrations of E2-6 : AL failed to elicit LLE similar to that produced by pheromone blend and produced only a BE (f). Scales in (Ab) (for all traces) = 1 sec, 20 mV. (B) Plots of the firing frequency, measured in 250-msec bins, of the neuron recorded in (A), including additional trials. In this and all subsequent plots, where only one concentration is specified, different symbols indicate separate trials at that concentration. Where different concentrations were tested, they are specified along with the corresponding symbol on the figure. The sequence of stimulus trials always proceeded from the lowest concentration to the highest. The time of delivery of the odor is indicated by a solid line beneath the traces or plots. (Kanzaki *et al.* [18]).

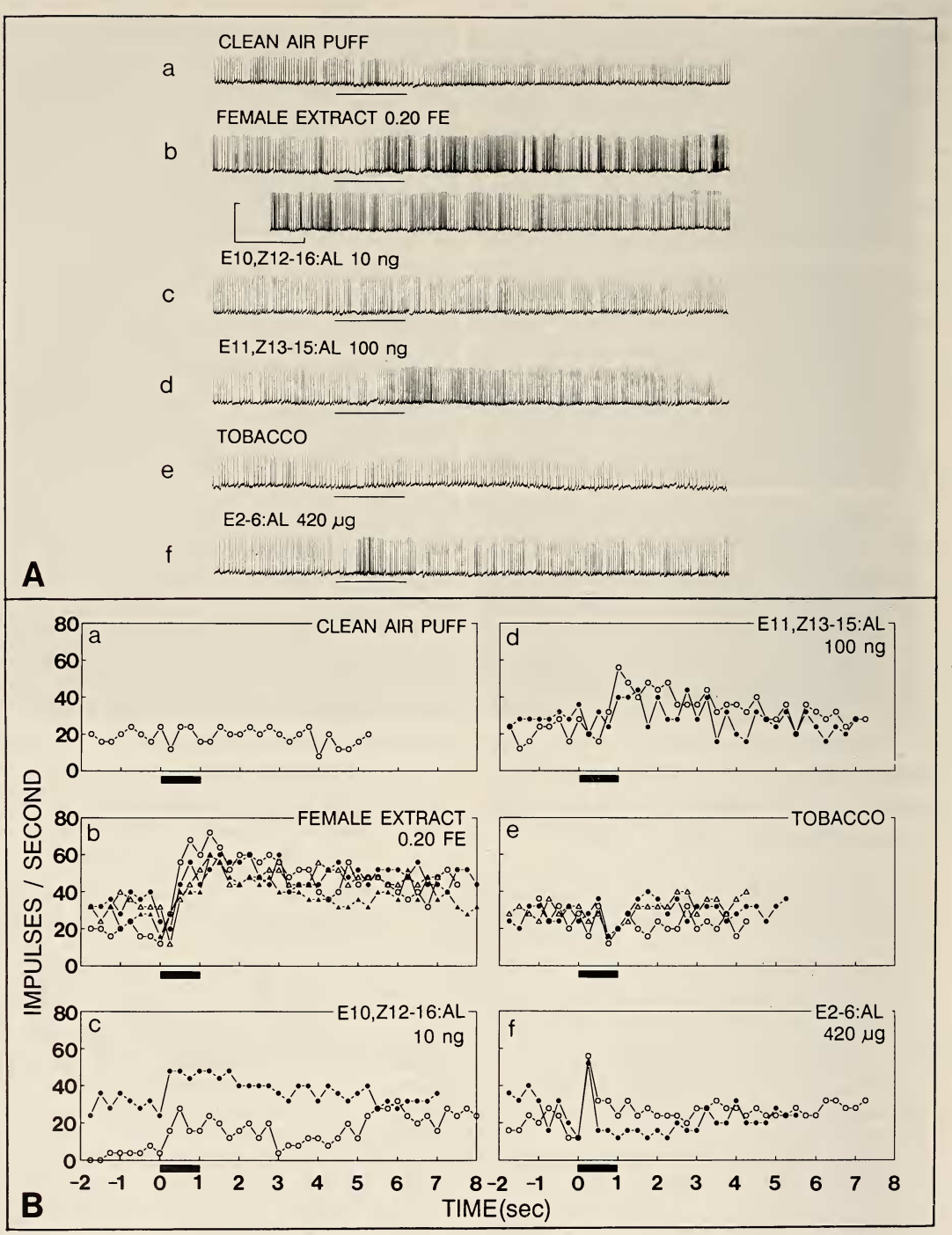
A
a
CLEAN AIR PUFF
b
FEMALE EXTRACT 0.20 FE
c
E10,Z12-16:AL 10 ng
d
E11,Z13-15:AL 100 ng
e
TOBACCO
f
E2-6:AL 420 µg
B
IMPULSES / SECOND
TIME(sec)
CLEAN AIR PUFF
FEMALE EXTRACT 0.20 FE
E11,Z13-15:AL 100 ng
TOBACCO
E10,Z12-16:AL 10 ng
E2-6:AL 420 µg

FIG. 8.

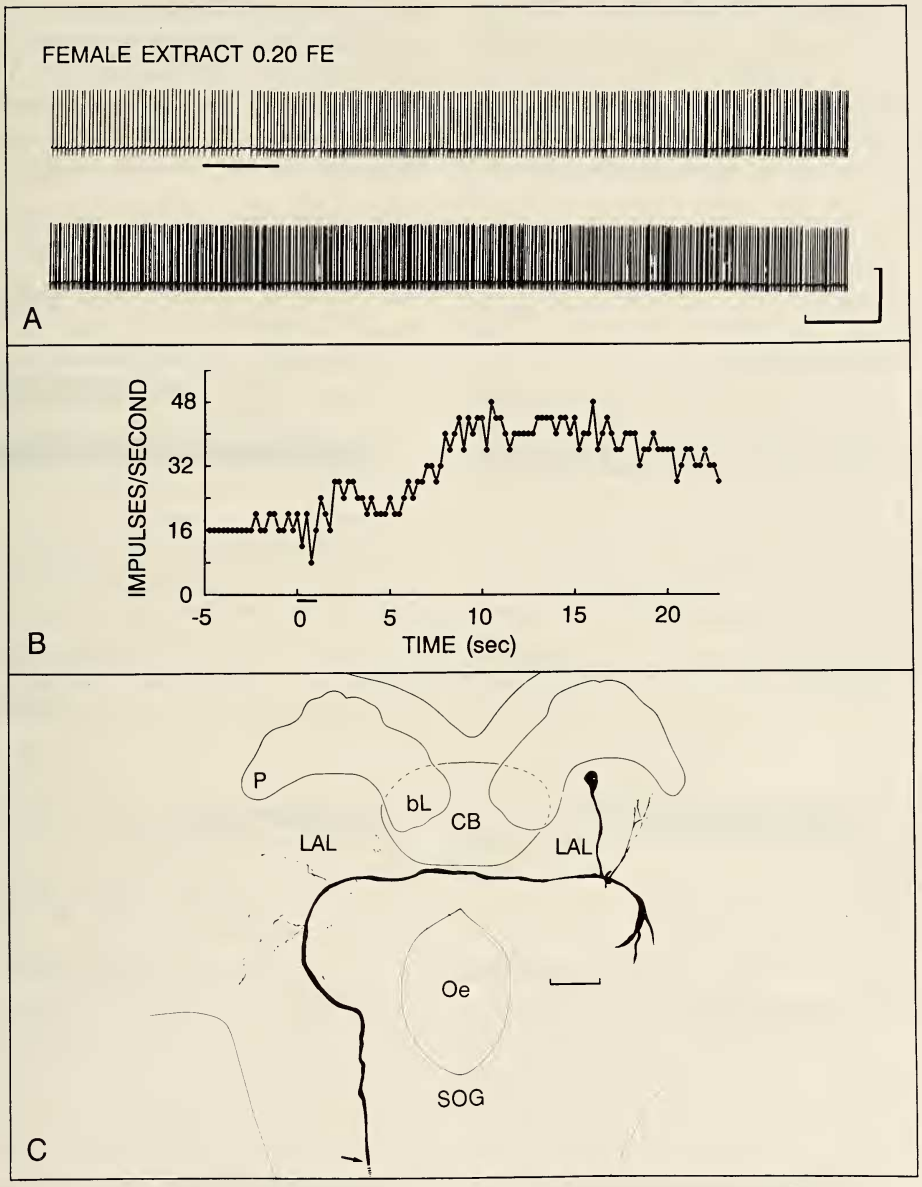

FIG. 9. Physiology and morphology of a descending neuron that responded to pheromonal stimuli with LLE. (A) Antennal stimulation with female extract elicited LLE lasting >20 sec (traces continuous). Scales = 1 sec, 40 mV. (B) Plot of the firing frequency of the neuron shown in (A) measured in 250-msec bins. (C) Reconstruction of the Lucifer Yellow-stained DN (in frontal view) from which the recordings shown in (A) were obtained. Bilateral processes of this neuron penetrated LALs on both sides of the brain and were linked by a large neurite that crossed the midline in the LAL commissure. The appearance of sparse branching of this neuron is probably a consequence of incomplete staining. The axon left the SOG in the connective contralateral to the soma (*arrow*) and could not be traced to the thoracic ganglia. The axon of this neuron had a diameter of about 8 μm and traveled in the dorsomedial quadrant of the contralateral VNC. Scale = 100 μm. (Kanzaki *et al.* [19]).

discriminate between the antennae if pheromonal stimuli are applied unilaterally (Figs. 9–11) [19].

(4) *Excitatory responses of olfactory PC neurons*

The majority of responses recorded in PC neurons to olfactory stimulation are excitatory. Although brief inhibition is sometimes elicited before LLE [18], sustained inhibition is rare. Recent immunocytochemical studies using antisera to GABA have revealed that numerous PC neurons, such as optic-lobe interneurons, negative feedback fibers of the MB, and fan-shaped neurons of the lower division of the central body, are GABA-immunoreactive [47]. Because GABA is believed

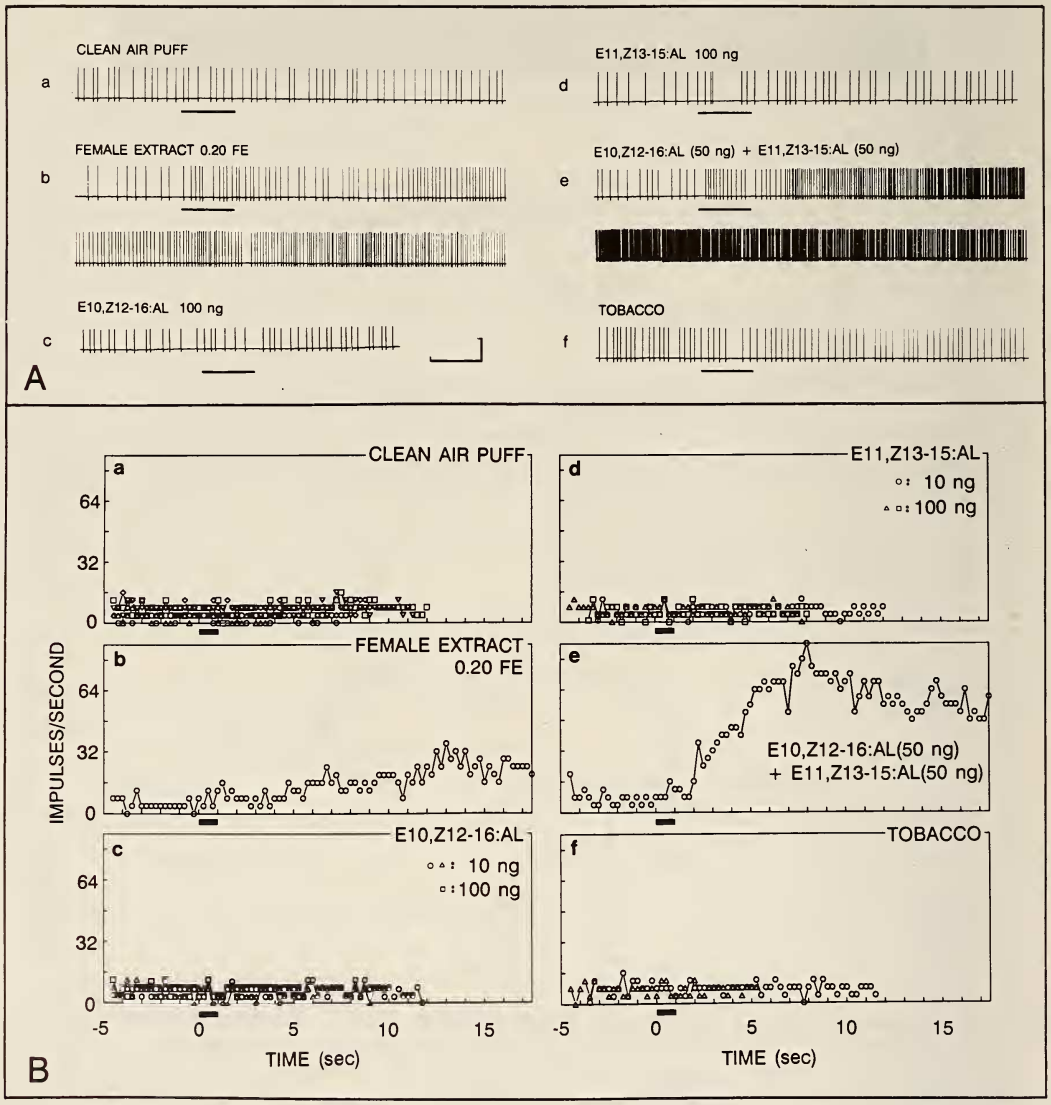

FIG. 10. LLE is elicited by pheromone blends but not by individual pheromone components. (A) LLE was elicited by stimulating with the complete wash of the female pheromone gland (b), or with blends of individual pheromone components (e), and it lasted much longer than the >15 sec shown in the two continuous traces in each trial. LLE could be elicited by stimulation of either antenna. Neither tobacco-leaf odor (f) nor individual pheromone components (c, d) activated the cell. Scales (for all traces)=1 sec, 40 mV. (B) Plots of the firing frequency, measured in 250-msec bins, of the neuron recorded in (A), including additional trials. (Kanzaki *et al.* [19]).

to be the neurotransmitter at many inhibitory synapses in the insect CNS [70–72], these are likely to be inhibitory elements. None of the olfactory PC neurons characterized morphologically thus far, however, resemble neurons revealed by GABA immunoreactivity or innervate the corresponding neuropils.

DESCENDING NEURONS

As described in protocerebral neurons, many olfactory PC neurons innervate a particular neuropil region, the lateral accessary lobe (LAL) in PC. LALs and certain adjacent neuropil regions are also innervated by branches of neurons that respond to olfactory stimuli and have axons that descend in the ventral nerve cord. It appears that the LALs may be interposed in the pathway of olfactory information flow from the AL through the lateral PC to the descending neurons (DNs) (Fig. 14). The activity of these DNs is of particular interest because the information they carry represents the integrated multimodal output of brain circuits that may act on thoracic motor circuitry to affect behavior.

(1) *Physiology of DNs*

Physioloigcal responses of olfactory DNs have several features in common with those of other neurons in the PC, but also have their own unique characteristics. As described for PC neurons, the responses of DNs to olfactory stimuli fall into two general classes, LLE and BE. The LLE-type responses are elicited preferentially in PC neurons by pheromonal stimuli, including individual pheromone components (Fig. 8), whereas in DNs, only blends of pheromone components or the female extract elicit LLE (Fig. 10) [19]. Although the responses of both PC neurons and DNs fit our definition of LLE, the activity of DNs typically takes longer to reach the peak firing rate than do the responses of PC neurons (3–10 sec for DNs vs. 1–3 sec for PC neurons) (Figs. 8–11). This slower rise to peak firing rates may be due to mixed excitatory and inhibitory drive during the early part of DN responses. DNs also typically continue to fire at high rates longer (>10 sec; Figs. 9–11) than PC neurons (<10 sec; Fig. 8). Some DNs show conditional responses in which identical pheromone-blend stimuli have different effects depending upon the state of firing of the DN (Fig. 11). Such conditional responses have not been encountered in olfactory neurons of either the AL or PC in *M. sexta* [9, 10, 18, 45, 48, 73] and *B. mori* [25–27, 49]. Conditional responses can be elicited in DNs by stimuli applied separately to either antenna [19]. PC neurons are sensitive only to stimuli applied to one of the antennae (Fig. 8) [18]. Integrated information about pheromone blends may be collected from circuitry on both sides of the brain by bilateral LAL neurons (Fig. 7). The arborizations of these bilateral neurons overlap with branches of DNs both within the LALs and in adjacent PC neuropils and may provide the anatomical substrate for synaptic interactions (Fig. 9). Neither direct structural contacts nor synaptic communications between these neurons have been demonstrated. However, we have not observed any neurons that might mediate the direct synaptic contacts between AL projection neurons and DNs, as has been suggested to occur in the lateral PC of cockroaches [31, 74].

(2) *Multimodality of DNs*

None of the olfactory DNs showed consistent responses to mechanosensory stimuli applied to either antenna individually [19]. About 50% of pheromone-unresponsive AL projection neurons [10] and about 7% of PC neurons [18] that responded to olfactory stimulation showed some responses to mechanosensory stimulation. Some DNs which carry mechanosensory information were characterized, but these neurons did not respond to any of the odorants tested (Kanzaki, unpublished data). By contrast, visual stimulation elicited excitatory or inhibitory responses in olfactory DNs. There were "on" or "off" responses in neurons showing LLE and reduction of spontaneous firing with tonic increases in illumination (Fig. 12) [19]. Neurons responding to olfactory stimuli with BE also show excitatory responses to lights on or off (Fig. 13) [19]. It is suggested that there is a stronger linkage between visual and olfactory modalities than between mechanosensory and olfactory modalities. The fact that sustained high illumination tended to inhibit back-

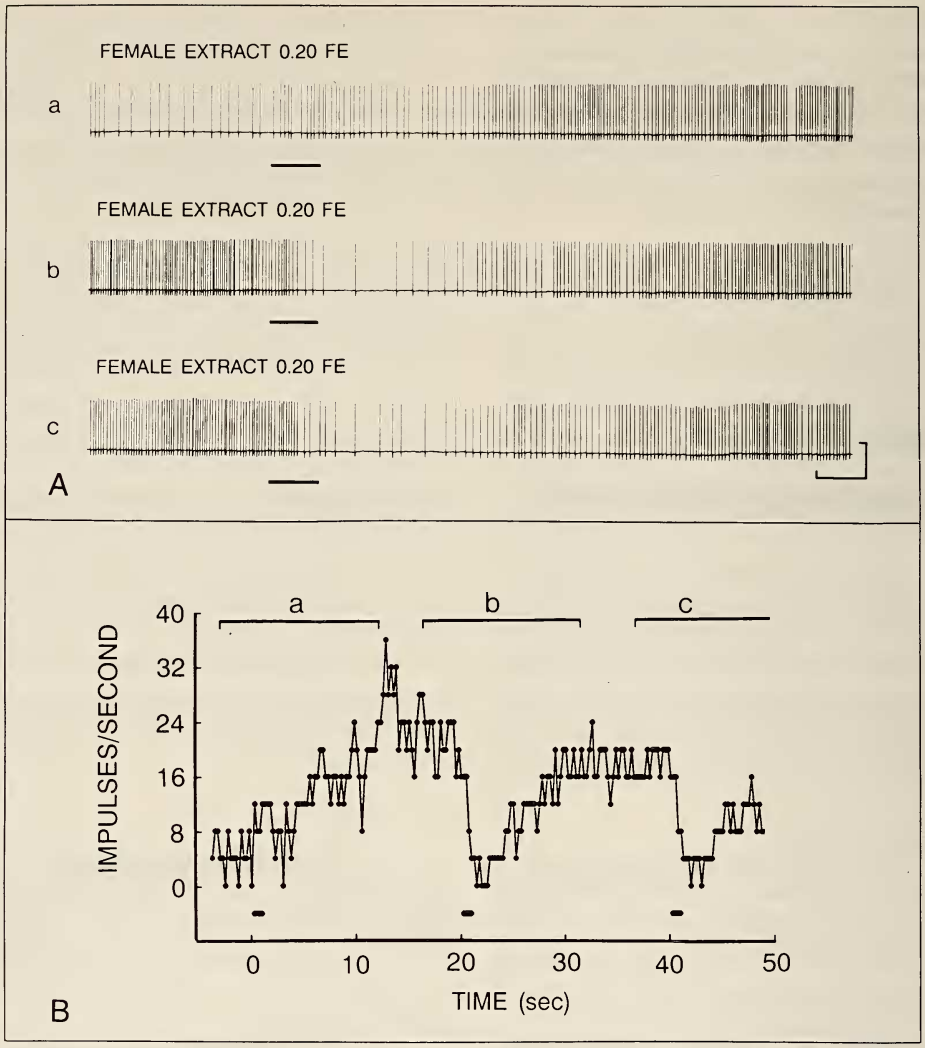

FIG. 11. State-dependent responses of a DN exhibiting LLE. (A) Responses to puffed stimulation with pheromone blend ipsilateral to the recording site in the cervical nerve cord. When the neuron was firing at a relatively low rate near 4 Hz, stimulation of the antenna with pheromone blend elicited LLE (a). When the neuron was in a state of high background firing due to the LLE elicited in (a), a similar stimulus applied to the same antenna caused a marked reduction in the firing rate (b) rather than further excitation. This inhibition of firing lasted only several seconds, and firing gradually returned to the previous higher rate. Repeated application of pheromone blend against the higher background firing rate again caused an inhibition (c). Scales = 1 sec, 40 mV. (B) Plot of the firing frequency of the neuron shown in (A). Bars above the plot indicate the segments corresponding to the traces in (A). Bars below the plot indicate times of stimulus application. (Kanzaki *et al.* [19]).

ground firing of DNs that process pheromonal information might be relevant to the observation that *M. sexta* males are inactive and unresponsive to pheromone blend in the daytime. Of course, many other parameters in addition to ambient light level change in the course of a circadian rhythm, e.g., levels of octopamine in the hemolymph [75].

In *Bombyx mori,* visual stimuli were effective in changing the firing state of certain interneurons [59]. Moreover, some of these neurons responded to both mechanosensory stimuli applied to the antennae and directional visual stimuli [59]. Pheromonal stimuli were shown to activate or amplify visual responses in several DNs of gypsy moth

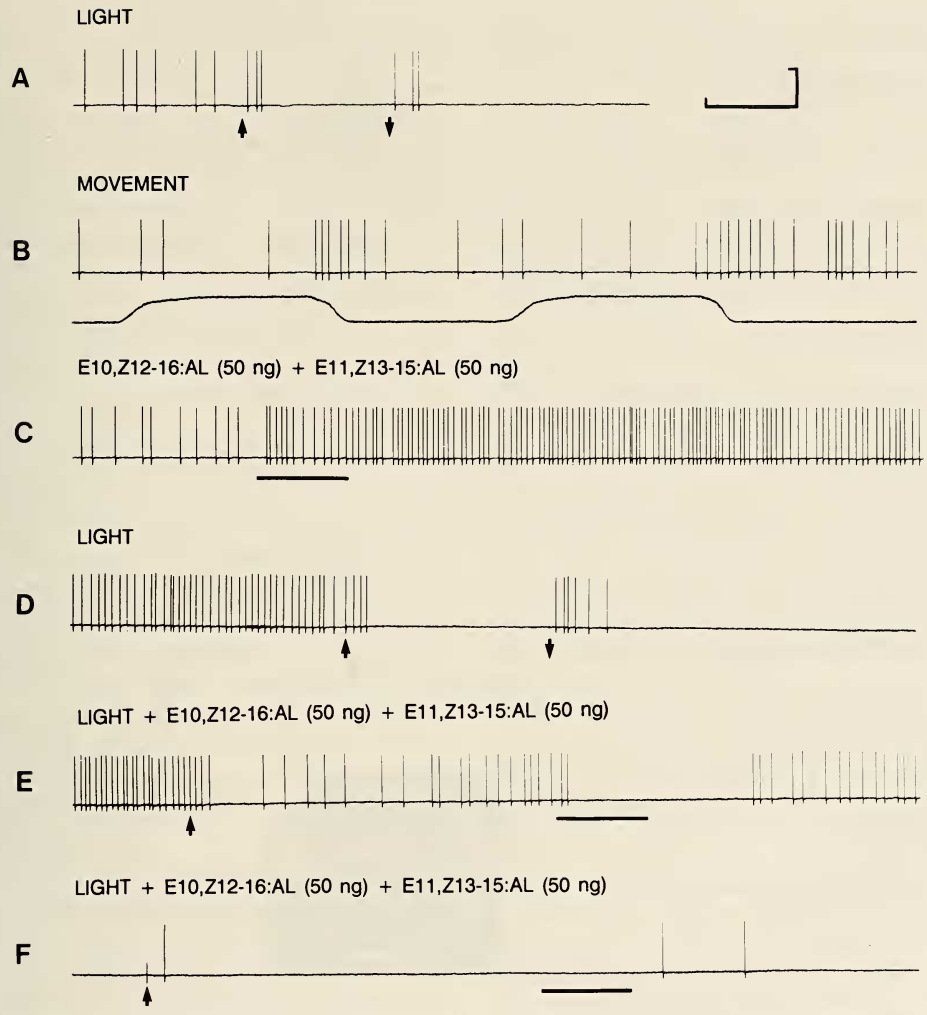

FIG. 12. Multimodal influences on a DN exhibiting LLE. (A) When this neuron was in a state of low background firing, turning a bright light on (*upward arrow*) and off (*downward arrow*) elicited brief on/off responses and led to suppression of tonic firing. (B) Moving a vertical black and white striped pattern horizontally in front of the moth with only dim illumination also elicited brief spiking responses that were directional. (Movement indicator trace below intracellular recording. *Upward deflection*=movement R to L, *downward deflection*=movement L to R). (C) Stimulation of the antenna with a blend of pheromone components (50 ng each of E10,Z12-16 : AL and E11,Z13-15 : AL) elicited LLE. (D) When the neuron was in a state of high background firing due to such LLE, increasing illumination (*upward arrow*) suppressed firing. Firing was not restored to the previous high rate even when illumination was reduced (*downward arrow*), although a brief "off" response was elicited. When the neuron was again firing at a high rate in a similar LLE activated by puffing the blend of pheromone components on the antenna (E), increasing illumination reduced but did not completely suppress firing of the cell. Another puff of the odor blend (50 ng each of E10,Z12-16 : AL and E11,Z13-15 : AL, *soild bar*) against this background of reduced firing caused suppression of firing lasting several seconds, whereupon the neuron gradually increased its firing rate to the pre-stimulus level. This response is consistent with the state-dependent responses elicited against the backdrop of LLE, as described above. (F) Increasing illumination (*upward arrow*) when the neuron was again in a state of low background firing produced an "on" response consisting of one spike and subsequently silenced the cell. Under this condition of high illumination, an odor stimulus similar to the one that previously excited the cell (e.g., c, d) (*solid bar*) failed to elicit LLE. Scales=1 sec, 40 mV. (Kanzaki *et al.* [19]).

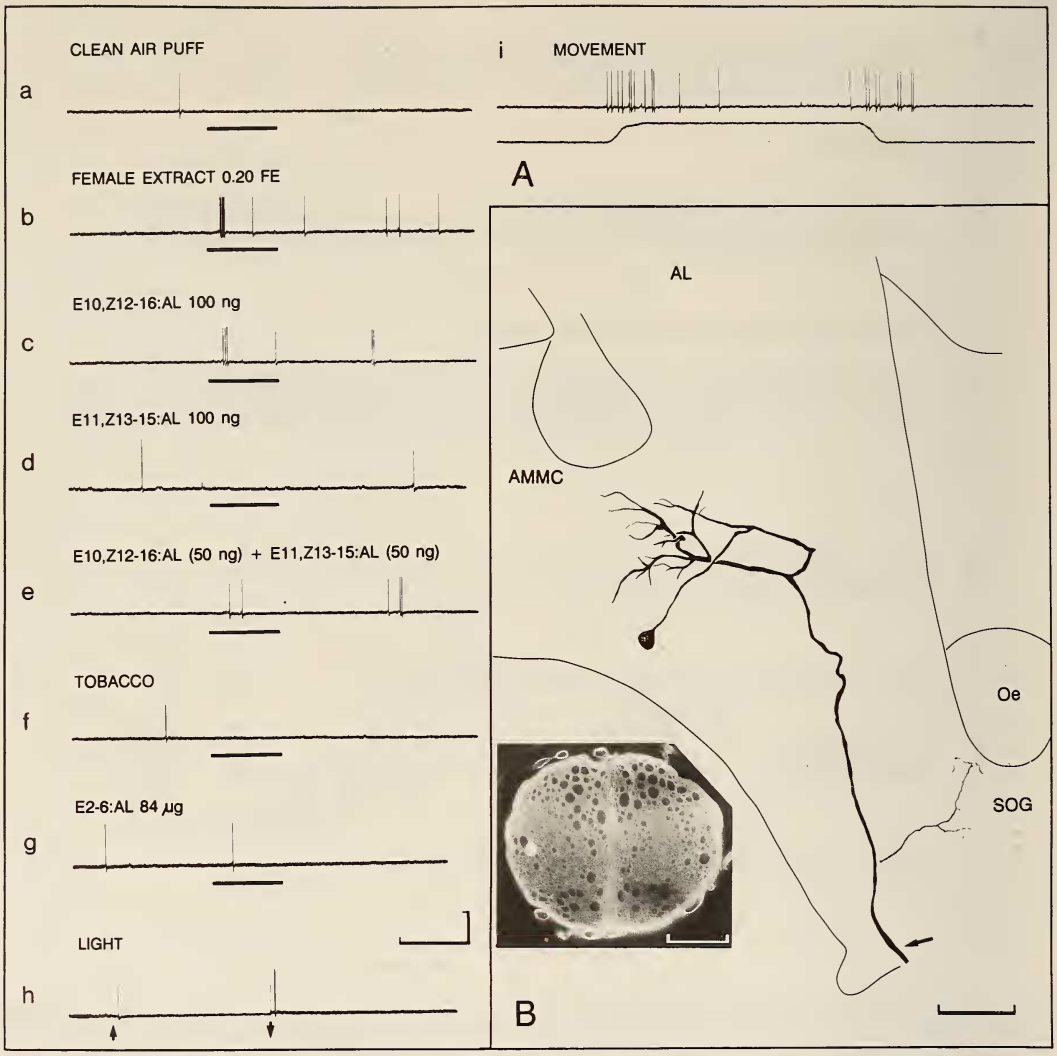

FIG. 13. Physiology and morphology of a unilateral descending neuron responding with BE. (A) Intracellularly recorded responses to odors and movement. This neuron exhibited an extremely low background level of activity. It gave only occasional signal action potentials when unstimulated or when clean air (a), plant odor (f), E2-6:AL (g), or pheromone components (d) were puffed onto the antenna. This neuron's failure to respond to these air-puff stimuli suggests an absence of sensitivity to gentle mechanical stimulation. Brief increases in firing at short latency, and slight increases in the occurrence of impulses at longer latencies, seemed to be elicited by female extract (b) and E10,Z12-16:AL (c, e). No responses were elicited by stimuli applied to the contralateral antenna. This neuron gave brief on/off responses consisting of one or two impulses when illumination was increased (h), and it gave a more vigorous response to movement of a pattern of vertical black and white stripes horizontally in front of the moth (i). Scales=1 sec, 40 mV. (B) Reconstruction (in frontal view) of the neuron whose responses are illustrated in (A). This neuron projected several stout branches to a region just ventral to the AMMC. The main neurite projected through the SOG ipsilaterally to the soma and sent off a fine branch there shortly before entering the ipsilateral cervical connnective (*arrow*). Inset: Cross section of the cervical connective showing the lateral position of the axon. Scale bars=100 µm. (Kanzaki *et al.* [19]).

Lymantria dispar [76].

(3) *Morphology of DNs*

Some DNs that show LLE or BE have branches in the LALs on either side (Fig. 9) [19]. Many pheromone-processing PC neurons innervate the LALs, and that some of these neurons provide the anatomical substrate for linking the LAL to the lateral PC [18], a region receiving axons of AL projection neurons. The LALs on the two sides of the brain are linked by bilateral PC neurons with arborizations in each LAL (Fig. 7) [18]. It appears that the LALs may be interposed in the pathway of olfactory information flow from the AL through the lateral PC to the DNs (Fig. 14).

Some DNs that show BE in response to pheromonal stimuli do not have neurites in the LALs (Fig. 13) [19]. Instead, their branches innervate a variety of regions within the ventral PC of the brain. Even in the bilateral neurons with dense arborizations in the LALs, some branches project out of the LALs to adjacent neuropil regions and could, in principle, provide a linkage to DNs that reside entirely outside of the LALs [19]. Alternatively, olfactory information may be delivered to these DNs by pathways that do not include LAL neurons (Fig. 13).

In general, LLE has been associated with neurons which contain branches within the LALs, while many cells exhibiting BE lack projections within these neuropil areas (Fig. 13) [19]. When tested with pheromonal stimuli applied to either antenna individually, the former neurons respond to stimuli applied to either side, while the latter are excited only by stimulation of the ipsilateral antenna (Fig. 13) [19]. Thus, it could be that multiple, higher-order pathways exist by which olfactory information reaches DNs. Some pathways may carry information confined to one side of the brain and may elicit BE in DNs that do not invade the LALs with their arborizations. Other pathways may lead to the LALs, where olfactory information from both sides of the brain is collected, and may elicit LLE in DNs with neurites in this neuropil area (Fig. 14) [19].

(4) *LLE responses in DNs*

Long-lasting increases in firing elicited by pheromone components have been described in a class of axons termed "flip-flopping" interneurons in male *B. mori* [59]. These neurons exhibited conditional responses, in that stimuli applied when a neuron was in a state of low-frequency firing elicited accelerated firing, while identical stimuli applied when the neuron was in a state of high-frequency firing caused decelerated firing (hence the term flip-flop). The conditional responses of *M. sexta* DNs differ from the flip-flopping of DN in *B. mori* in several ways. In *B. mori,* long-lasting increases in firing could be activated in flip-flopping neurons by the primary component of the pheromone blend, bombykol, which also activates the entire sequence of zig-zag walking by which males approach signalling females [59]. In *M. sexta* individual pheromone components fail to elicit LLE, but blends of the major pheromone components or female extract are effective (Fig. 10) [19]. In *B. mori*, both high and low states of firing elicited by pheromone components were stable and lasted as long as 4 min. In *M. sexta,* LLE elicited by pheromone blends can last several tens of seconds, but the conditional reduction of firing elicited by subsequent pheromone-blend stimuli spontaneously reverts to the state of high frequency firing after several seconds (Fig. 11) [19].

The activity states of flip-flopping neurons of *B. mori* were correlated with changes in antennal posture that occur in association with turns during the olfactory-mediated zig-zag walking [59].

Another group of DNs has been identified in *B. mori* that showed LLE responses to stimulation of antenna by bombykol but did not give conditional responses similar to flip-flopping [77]. The dose-response relationship between bombykol stimuli and prolonged firing of these DNs closely resembled a similar relationship between pheromonal stimuli and production of wing fluttering. Kanzaki and Shibuya [77] suggested that these neurons may have a role in initiation and maintenance of the "mating dance" of *B. mori*. The morphology of these neurons was shown by dye injection to be very similar to that of the *M. sexta* neurons illustrated in Fig. 9C [77].

The responses recorded in DNs may play a role in central processing of olfactory information or in

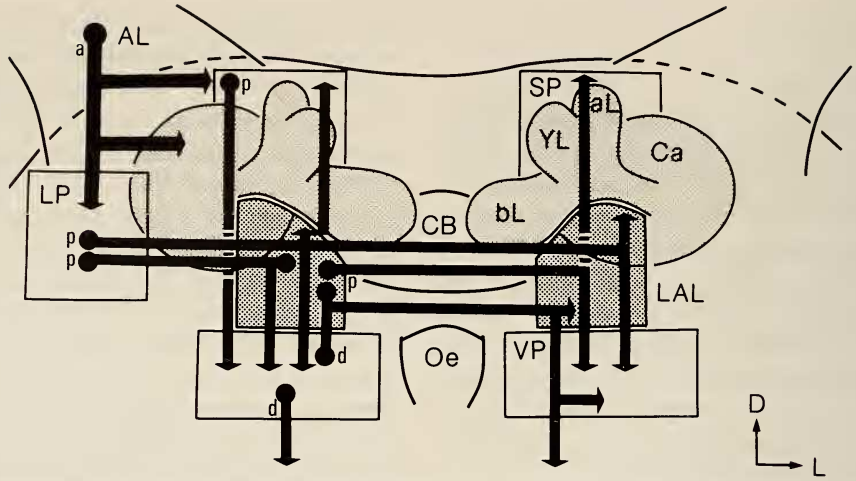

FIG. 14. Schematic summary of higher-order olfactory pathways in the brain of male moth *Manduca sexta*. This diagram summarizes functional pathways for flow of olfactory information through the brain derived from the morphologies of pheromone-sensitive interneurons that were revealed by Lucifer Yellow injection. Frontal view. Output neurons (*AL PNs*) of the antennal lobes (*AL*), the primary olfactory neuropil, are depicted by lines with a letter "a". Lines with a latter "p" depict olfactory neurons intrinsic to the protocerebrum. Descending neurons are represented in lines with a letter "d". Boxes outlined by lines depict prominent target neuropil areas in the protocerebrum defined by the projection patterns of olfactory neurons. *LP*; lateral protocerebrum, *SP*; superior protocerebrum, *VP*; ventral protocerebrum.

generating or controlling olfactory mediated behavior. The LLE and state dependent activity changes are particularly intriguing, because they seem to represent an essential link towards behavior. These neurons are premotor DNs to the motor circuits of the thorax and their responses are elicited by complex and behaviorally relevant stimuli, i.e., the pheromone blend rather than individual pheromone components [19].

Analysis of upwind and casting flight behavior in several moth species by Baker's group has led to a recent proposal that the goal-oriented upwind flight is mediated by the complementary action of a dual flight control system [78–81]. In this system, contact with pheromone-laden parcels of air in the turbulent plume would produce (1) rapidly activated surges of upwind flight that would also be rapidly terminated upon the next encounter with clean air, and (2) long-lasting activation of a counterturning program which would continue to produce turns that comprise the "casting flight" that persists for seconds when the moth loses contact with the pheromone plume. Our finding that the responses of higher-order olfactory neurons, including descending neurons, fall into the general categories of phasic (BE) or tonic (LLE) activity invites the suggestion that these classes of neurons may separately drive the phasic (upwind surges) and tonic (counterturning) components of the dual flight control system [78–81].

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